



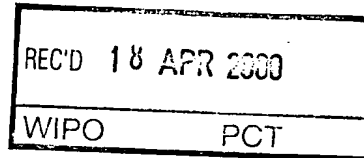
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4.	Title of the invention	HUMAN AKT-3		
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HUMAN AKT-3

The present invention is concerned with cloning and expression of a new human serine/threonine kinase
5 termed "Akt-3" and, in particular, with nucleic acid molecules encoding the Akt-3 protein, the protein itself and compounds which can be used to inhibit cell survival, following removal of a cell from survival factors, such as, an extracellular matrix (anoikis).

10 A characteristic feature of many cancer cells is their ability to grow independently of adhesion. In contrast, when untransformed endothelial cells are prevented from adhering to the extracellular matrix
15 (ECM), they undergo apoptosis (Frisch & Francis, 1994; Meredith et al, 1993). The process by which normally adherent cells are triggered to undergo apoptosis when they are unable to adhere to ECM has been termed
20 "anoikis" (Frisch & Ruoslahti, 1997) and is an example of the effect on a cell of removal of a survival factor. Changes in signalling by adhesion molecules can lead to resistance to anoikis (Frisch & Ruoslahti
25 1997) and this may contribute to the mechanism whereby cancer cells that grow independently of adhesion are able to avoid anoikis.

Akt (also known as protein kinase B (PKB) or 'related to A and C protein kinase' (RAC-PK)) is a
30 serine/threonine kinase that has been implicated in regulating cell survival (Khwaja et al., 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Datta et al., 1997, Marte & Downward,
1997). Akt can inhibit apoptosis induced by detachment from ECM (anoikis; Khwaja et al., 1997), as well as by
35 survival factor withdrawal (Kennedy et al., 1997;

Ahmed et al., 1997; Dudek et al., 1997; Kauffman-Zeh et al., 1997; Philpott et al., 1997; Crowder & Freeman, 1998; Eves et al., 1998) or irradiation (Kulik et al., 1997).

5

Akt comprises an NH₂-terminal pleckstrin homology (PH) domain involved in lipid binding, a kinase domain and a COOH-terminal 'tail'. Akt is thought to be activated by recruitment to the plasma membrane and subsequent phosphorylation by two upstream kinases, PDK-1 and PDK-2 (reviewed in Coffey et al., 1998; Alessi & Cohen, 1998). The binding of 3-phosphoinositides, generated by phosphatidylinositol 3-kinase (PI 3-kinase), to the PH domain of Akt is believed to promote translocation to the plasma membrane and to facilitate phosphorylation of Akt-1 by PDK-1 at Thr³⁰⁸ (Alessi et al., 1996; Alessi et al., 1997; Stephens et al., 1998) or of Akt-2 at Thr³⁰⁹ (Meier et al., 1997). In addition to phosphorylation of Thr³⁰⁸, full activation requires phosphorylation of the COOH tail at Ser⁴⁷³ in Akt-1 (Alessi et al., 1996) or at Ser⁴⁷⁴ in Akt-2 (Meier et al., 1997). The enzyme responsible for phosphorylation of Ser⁴⁷³/Ser⁴⁷⁴ was originally named PDK-2 but recently the integrin-linked kinase, ILK (Delcommenne et al., 1998) has emerged as a candidate for this function.

Two human isoforms of Akt have been described to date, Akt-1 and Akt-2 (Coffey & Woodgett, 1991; Jones et al., 1991; Cheng et al., 1992). A third isoform, here referred to as Akt-3, has been described in the rat (Konishi et al., 1995). Since this rat Akt-3 possesses an apparently truncated tail and thereby lacks Ser⁴⁷³, its regulation may differ from that of Akt-1 and Akt-2. Both Akt-1 and Akt-2 are expressed widely, although the expression of Akt-2 is most prominent in insulin-

responsive tissues, such as liver and skeletal muscle (Konishi et al., 1994; Altomare et al., 1995). Akt-1 and Akt-2 are activated by insulin in rat adipocytes, hepatocytes and skeletal muscle. In contrast, Akt-3
5 does not appear to be strongly activated by insulin in these tissues (Walker et al., 1998). The role of the various Akt isoforms in insulin signalling may limit the utility of compounds that inhibit Akt-1 or Akt-2 activity as such agents may induce symptoms observed
10 in patients with diabetes. We hypothesized that this problem may be avoided by using selective inhibitors of Akt-3 and this prompted us to identify the human analogue of rat Akt-3.

15 The present inventors have now identified and characterised a nucleic acid molecule that encodes the human isoform of Akt-3. Significantly, human Akt-3 possesses a COOH-terminal tail that contains an amino acid residue analogous to Ser⁴⁷³/Ser⁴⁷⁴ previously
20 implicated in the activation of Akt-1/Akt-2, but absent in the rat Akt-3 protein.

Therefore, there is provided by a first aspect of the present invention a nucleic acid molecule encoding
25 human Akt-3 or a functional equivalent or bioprecursor thereof, comprising the amino acid sequence illustrated in Figure 2. Preferably, the molecule is a DNA molecule and even more preferably a cDNA molecule, and even more preferably comprises the
30 sequence of nucleotides illustrated in Figure 1. Also provided by this aspect of the invention is a nucleic acid molecule capable of hybridising to the molecule according to the invention under high stringency conditions.

Stringency of hybridisation as used herein refers to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. T_m can be approximated by the formula:

$$81.5^{\circ}\text{C} + 16.6(\log_{10}[\text{Na}^+] + 0.41 (\% \text{G\&C}) - 6001/l$$

wherein l is the length of the hybrids in nucleotides. T_m decreases approximately by 1-1.5°C with every 1% decrease in sequence homology.

The nucleic acid capable of hybridising to nucleic acid molecules according to the invention will generally be at least 85%, preferably at least 90% and more preferably at least 95% homologous to the nucleotide sequences according to the invention.

The DNA molecules according to the invention may, advantageously, be included in a suitable expression vector to express polypeptides encoded therefrom in a suitable host.

The present invention also comprises within its scope proteins or polypeptides encoded by the nucleic acid molecules according to the invention or a functional equivalent, derivative or bioprecursor thereof.

An expression vector according to the invention includes a vector having a nucleic acid according to the invention operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship

permitting them to function in their intended manner. Such vectors may be transformed into a suitable host cell to provide for expression of a polypeptide according to the invention. Thus, in a further
5 aspect, the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell, transformed or transfected with an expression vector as described above under conditions to provide for expression by
10 the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

The vectors may be, for example, plasmid, virus or
15 phage vectors provided with an origin of replication, optionally a promoter for the expression of said nucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable markers, such as, for example, ampicillin resistance.

20 Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector
25 may include a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a
heterologous or homologous promoter for RNA polymerase
30 II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art.

35

A nucleic acid molecule according to the invention may be inserted into the vectors described in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense
5 nucleic acids may be produced by synthetic means.

In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any minor base variations including in
10 particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the degenerate code in conservative amino acid substitutions. The term
"nucleic acid sequence" also includes the
15 complementary sequence to any single stranded sequence given regarding base variations.

The present invention also advantageously provides nucleic acid sequences of at least approximately 10
20 contiguous nucleotides of a nucleic acid according to the invention and preferably from 10 to 50 nucleotides. These sequences may, advantageously be used as probes or primers to initiate replication, or the like. Such nucleic acid sequences may be produced
25 according to techniques well known in the art, such as by recombinant or synthetic means. They may also be used in diagnostic kits or the like for detecting the presence of a nucleic acid according to the invention. These tests generally comprise contacting the probe
30 with the sample under hybridising conditions and detecting for the presence of any duplex or triplex formation between the probe and any nucleic acid in the sample.

35 According to the present invention these probes may be

anchored to a solid support. Preferably, they are present on an array so that multiple probes can simultaneously hybridize to a single biological sample. The probes can be spotted onto the array or synthesised *in situ* on the array. (See Lockhart et al., Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation to high density oligonucleotide arrays". A single array can contain more than 100, 500 or even 1,000 different probes in discrete locations.

Advantageously, the nucleic acid sequences, according to the invention may be produced using such recombinant or synthetic means, such as for example using PCR cloning mechanisms which generally involve making a pair of primers, which may be from approximately 10 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with mRNA, cDNA, or genomic DNA from a human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in the art, such as described in Sambrook et al (Molecular Cloning: a Laboratory Manual, 1989).

The nucleic acids or oligonucleotides according to the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S , enzyme labels or other protein labels such as biotin or fluorescent markers. Such labels may be added to the nucleic acids or oligonucleotides of the invention and may be detected using known techniques *per se*.

A further aspect of the invention comprises human Akt-3 or a functional equivalent, derivative or bioprecursor thereof, comprising an amino acid sequence as illustrated in Figure 2.

5

The polypeptide designated human Akt-3 according to the invention includes all possible amino acid variants encoded by the nucleic acid molecule according to the invention including a polypeptide encoded by said molecule and having conservative amino acid changes. Polypeptides according to the invention further include variants of such sequences, including naturally occurring allelic variants which are substantially homologous to said polypeptides. In this context, substantial homology is regarded as a sequence which has at least 90% amino acid homology with the polypeptides encoded by the nucleic acid molecules according to the invention.

20 The nucleic acid molecule or the human Akt-3 according to the invention may, advantageously, be used as a medicament or in the preparation of a medicament, for treating diseases associated with Akt-3 expression, such as, cancer or the like.

25

Advantageously, the nucleic acid molecule or the polypeptide according to the invention may be provided in a pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

30

The present invention is further directed to inhibiting Akt-3 *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation

35

or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the mature protein sequence, which encodes for the protein of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee et al. Nucl. Acids Res., 6:3073 (1979); Cooney et al., Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991), thereby preventing transcription and the production of Akt-3. The antisense RNA oligonucleotide hybridises to the mRNA *in vivo* and blocks translation of an mRNA molecule into the Akt-3 (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)).

Alternatively, the oligonucleotide described above can be delivered to cells by procedures in the art such that the anti-sense RNA or DNA may be expressed *in vivo* to inhibit production of Akt-3 in the manner described above.

Antisense constructs to Akt-3, therefore, may inhibit the survival promoting activity of the cell following removal of the survival factors and prevent further cancer or tumour growth.

According to a further aspect of the invention there is also provided a transgenic cell, tissue or organism comprising a transgene capable of expressing human Akt-3 protein according to the invention. The term "transgene capable of expression" as used herein means

a suitable nucleic acid sequence which leads to expression of human Akt-3 or human proteins having the same function and/or activity. The transgene, may include, for example, genomic nucleic acid isolated
5 from human cells or synthetic nucleic acid, including DNA integrated into the genome or in an extrachromosomal state. Preferably, the transgene comprises the nucleic acid sequence encoding the proteins according to the invention as described
10 herein, or a functional fragment of said nucleic acid. A functional fragment of said nucleic acid should be taken to mean a fragment of the gene comprising said nucleic acid coding for the proteins according to the invention or a functional equivalent, derivative or a
15 non-functional derivative such as a dominant negative mutant, or bioprecursor of said proteins. For example, it would be readily apparent to persons skilled in the art that nucleotide substitutions or deletions may be used using routine techniques, which
20 do not affect the protein sequence encoded by said nucleic acid, or which encode a functional protein according to the invention.

Human Akt-3 protein expressed by said transgenic cell,
25 tissue or organism or a functional equivalent or bioprecursor of said protein also form part of the present invention.

Antibodies to human Akt-3 may, advantageously, be
30 prepared by techniques which are known in the art. For example, polyclonal antibodies may be prepared by inoculating a host animal, such as a mouse, with human Akt-3 according to the invention or an epitope thereof and recovering immune serum. Monoclonal antibodies may
35 be prepared according to known techniques such as

described by Kohler R. and Milstein C., Nature (1975)
256, 495-497.

5 Antibodies according to the invention may also be used
in a method of detecting for the presence of human
Akt-3 according to the invention, which method
comprises reacting the antibody with a sample and
identifying any protein bound to said antibody. A kit
10 may also be provided for performing said method which
comprises an antibody according to the invention and
means for reacting the antibody with said sample.

15 Proteins which interact with the polypeptide of the
invention may be identified by, for example,
investigating protein-protein interactions using the
two-hybrid vector system first proposed by Chien et al
(1991).

20 This technique is based on functional reconstitution
in vivo of a transcription factor which activates a
reporter gene. More particularly the technique
comprises providing an appropriate host cell with a
DNA construct comprising a reporter gene under the
control of a promoter regulated by a transcription
25 factor having a DNA binding domain and an activating
domain, expressing in the host cell a first hybrid DNA
sequence encoding a first fusion of a fragment or all
of a nucleic acid sequence according to the invention
and either said DNA binding domain or said activating
30 domain of the transcription factor, expressing in the
host at least one second hybrid DNA sequence, such as
a library or the like, encoding putative binding
proteins to be investigated together with the DNA
binding or activating domain of the transcription
35 factor which is not incorporated in the first fusion;

detecting any binding of the proteins to be
investigated with a protein according to the invention
by detecting for the presence of any reporter gene
product in the host cell; optionally isolating second
5 hybrid DNA sequences encoding the binding protein.

An example of such a technique utilises the GAL4
protein in yeast. GAL4 is a transcriptional activator
of galactose metabolism in yeast and has a separate
10 domain for binding to activators upstream of the
galactose metabolising genes as well as a protein
binding domain. Nucleotide vectors may be
constructed, one of which comprises the nucleotide
residues encoding the DNA binding domain of GAL4.
15 These binding domain residues may be fused to a known
protein encoding sequence, such as for example the
nucleic acids according to the invention. The other
vector comprises the residues encoding the protein
binding domain of GAL4. These residues are fused to
20 residues encoding a test protein. Any interaction
between polypeptides encoded by the nucleic acid
according to the invention and the protein to be
tested leads to transcriptional activation of a
reporter molecule in a GAL-4 transcription deficient
25 yeast cell into which the vectors have been
transformed. Preferably, a reporter molecule such as
 β -galactosidase is activated upon restoration of
transcription of the yeast galactose metabolism genes.

30 A further aspect of the invention provides a method of
identifying compounds which selectively inhibit human
Akt-3 mediated promotion of cell survival upon
withdrawal of survival factors, such survival factors
including but not being limited to extracellular
35 matrix and IgF-1, said method comprising i) providing

on the one hand a cell that survives in the presence of a survival factor but not in the absence of said survival factor unless said cell is transformed with an expression vector according to the invention in addition to providing as a control a cell which in the presence of survival factor has not been transformed with said vector, ii) contacting each of said cells with a test compound to ensure said compound is not toxic to said cells, iii) subsequently contacting said cells with the test compound following removal of said cells from said survival factors, wherein death of said cell when transformed with said expression vector is indicative of selective inhibition of said compound on the survival promoting human Akt-3 pathway or a parallel pathway.

Compounds which are identified according to this aspect of the invention in addition to antibodies to the human Akt-3 may, advantageously, be utilised as a medicament or alternatively in the preparation of a medicament for treating diseases associated with expression of human Akt-3 protein according to the invention.

The present invention may be more clearly understood with reference to the following example which is purely exemplary and the accompanying drawings wherein:

Figure 1 is an illustration of the cDNA sequence and deduced amino acid sequence of human Akt-3. The Akt-3 coding sequence and parts of the 5' and 3' untranslated regions are shown and numbered in the left hand column. The deduced amino acid sequence of the Akt-3 protein is shown above the corresponding DNA sequence

and is numbered in the right hand column. The two amino acid residues that are presumed to be phosphorylated upon activation of Akt-3 (Thr³⁰⁵ and Ser⁴⁷²) are in bold and marked with an asterisk. The COOH-terminal part of the human Akt-3 protein that differs with the rat homologue is underlined.

Figure 2 is an alignment of the deduced amino acid sequences for human Akt-1, Akt-2 and Akt-3. The sequences were aligned using the ClustalW alignment program (EMBL, Heidelberg, Germany). Amino acid residues conserved between all three proteins are included in the black areas. Residues conserved between only two of the sequences are shaded in grey. Amino acid residues are numbered in the right hand column. The conserved Thr and Ser residues that are presumed to be phosphorylated upon activation are marked with an asterisk above the sequence.

Figure 3 is an illustration of phosphorylation of histone H2B by Akt-3 variants. (A) Akt-3 was expressed as a GST fusion protein in *E. Coli*. To assess hAkt-3 activity, Histone H2B was incubated with GST-Akt-3 and GST-Akt-3 variants for the indicated time and the extent of phosphorylation assessed after SDS-PAGE. The variants of Akt-3 are designated: W.T., wild type; T305D, Thr³⁰⁵ mutated to Asp; S472D, Ser⁴⁷² mutated to Asp; T305D,S472D, both Thr³⁰⁵ and Ser⁴⁷² mutated to Asp. No significant phosphorylation was observed when GST was used in place of GST-Akt. The results are the mean (\pm s.e.m.; n = 3 to 6) and are expressed relative to the extent of phosphorylation of H2B catalysed by T305D,S472D hAkt-3 after 45 minutes. Insert, The purity of the purified GST (lane1), wild-type Akt-3 (lane 2), T305D Akt-3 (lane 3), S472D Akt-3 (lane 4)

or T305DS472D Akt-3 (lane 5) was assessed by SDS-PAGE and by Coomassie blue staining. (B) Cos-7 cells were transfected with either vector (lanes 1 & 2) or Akt-3 (lanes 3 & 4) and either treated with buffer (lanes 1 & 3) or IGF-1 (10 ng/ml; lanes 2 and 4). Akt-3 was immunoprecipitated with antibody 12CA5 (anti-HA tag) and phosphorylation of histone 2B measured. The result shown is representative of two experiments.

Figure 4 is an illustration of inhibition of Akt-3 by staurosporine and R0 31-8220. Histone H2B was treated with Akt-3 (T305D,S472D variant) in the presence of the indicated concentrations of either staurosporine or Ro 31-8220. After 30 minutes, the reaction was terminated and the extent of H2B phosphorylation quantified on a phosphorimager following SDS-PAGE. The results (mean \pm s.e.m., n=3) are expressed as relative to (%) the phosphorylation observed in the presence of solvent (control, "C").

Figure 5 is an illustration of chromosomal localisation of human Akt-3. Diagram of FISH mapping results for Akt-3. Each dot represents the double FISH signals detected on human chromosome 1, region q43-q44. Example of FISH mapping of Akt-3. The left panel shows the FISH signals on chromosome 1. The right panel shows the same mitotic figure stained with 4',6-diamidino-2-phenylindole to identify chromosome 1.

Figure 6 is an illustration of expression of Akt-3 in different human tissues. (A) Northern blot analysis of tissue expression of Akt-3. The expression of hAkt-3 mRNA in different human tissues was assessed using a probe corresponding to the 3' untranslated region of hAkt-3 to analyse a blot of human polyA⁺ RNA

("Multiple Tissue Northern"). Human β -actin was used as a control to confirm equal loading of the lanes (data not shown). (B) and (C) RT-PCR analysis of tissue expression of Akt-3. RT-PCR analyses were performed on cDNA from different human tissues (B) and from different tumor cell lines (C) using primers specific for human Akt-3 or G3PDH (control) for the indicated number of PCR cycles. Bands of the expected size (425 bp for Akt-3 and 1 kb for G3PDH) are visible on the gels. The images from the ethidium bromide stained 1.2% agarose gels were inverted for clarity using the EagleSight software (Stratagene). The results from similar PCR reactions performed for 25, 30 or 35 cycles are not shown but indicated that the results from this figure are in the linear range of amplification. Caco-2 = colorectal adenocarcinoma; T-84 = colorectal carcinoma; MCF-7 = breast adenocarcinoma; T-47D = breast ductal gland carcinoma; HT1080 = bone fibrosarcoma; SaOS-2 = osteosarcoma; SK-N-MC = neuroblastoma; HepG2 = hepatoblastoma; JURKAT = T-cell leukemia.

MATERIALS AND METHODS

25 **Oligonucleotide synthesis and DNA sequence determination**

All primers were obtained from Eurogentec, Seraing, Belgium. Insert-specific sequencing primers (15- and 16-mers) were designed by visual inspection of the DNA sequences. DNA was prepared on Qiagen-tip-20 columns or on Qiaquick spin columns (Qiagen GmbH, Düsseldorf, Germany) and recovered from the spin columns in 30 μ l Tris/EDTA-buffer (10mM TrisHCl pH 7.5, 1 mM EDTA (sodium salt)). Sequencing reactions were performed

using BigDye™ Terminator Cycle Sequencing Ready
Reaction kits (Perkin Elmer, ABI Division, Foster
City, CA, USA) and were run on an Applied Biosystems
377 DNA sequencer (Perkin Elmer, ABI Division, Foster
5 City, CA, USA).

Molecular cloning of human Akt-3.

Using the rat RAC-PK γ sequence (Konishi et al., 1995;
10 GenBank acc. no. D49836) as a query sequence, a BLAST
(Basic Local Alignment Search Tool; Altschul et al.,
1990) search was carried out in the WashU Merck
expressed sequence tag (EST) database (Lennon et al.,
1996) and in the proprietary LifeSeq™ human EST
15 database (Incyte Pharmaceuticals Inc, Palo Alto, CA,
USA). Several human EST clones with high similarity to
the rat RAC-PK γ were identified. One EST sequence
(Incyte accession number 2573448) derived from a
hippocampal cDNA library, contained part of the coding
20 sequence including the putative methionine start codon
(ATG) and part of the 5' untranslated region. The
start codon was surrounded by a Kozak consensus
sequence for translation start and an in-frame stop
codon was present at positions -6 to -3. Based on this
25 239 bp sequence, oligonucleotide sense primers were
synthesised for 3' rapid amplification of cDNA ends (3'
RACE) experiments: Akt-3sp1 = 5'-ACC ATT TCT CCA AGT
TGG GGG CTC AG-3' and Akt-3sp2 = 5'-GGG AGT CAT CAT GAG
CGA TGT TAC C-3'. 3' RACE experiments were performed on
30 human fetal brain or human cerebellum Marathon-Ready™
cDNA (Clontech Laboratories, Palo Alto, CA, USA)
according to manufacturer's instructions using Akt-
3sp1/race-ap1 as primers in the primary PCR and Akt-
3sp2/race-ap2 in the nested PCR. Resulting PCR
35 fragments were cloned and sequenced. This extended the

Akt-3 coding sequence by 916 bp, but the novel sequence did not include an in-frame stop codon. A second round of 3' RACE amplification was performed on human brain Marathon Ready™ cDNA using sense primers based on the sequence obtained in the first round (Akt-3sp3 = 5'-CAC TCC AGA ATA TCT GGC ACC AGA GG-3' and Akt-3sp4 = 5'-CTA TGG CCG AGC AGT AGA CTG GTG G-3') in combination with race-ap1 and race-ap2, respectively. Now, the obtained sequence included an in-frame stop codon and the 3' untranslated sequence up to the poly(A) tail. Antisense primers were designed based on the 3' untranslated region (Akt-3ap4 = 5'-TGC CCC TGC TAT GTG TAA GAG CTA GG-3' and Akt-3ap5 = 5'-AAG AGC TAG GAC TGG TGA TGT CCA GG-3') and the complete Akt-3 coding sequence was amplified from human hippocampal cDNA using Akt-3sp1/Akt-3ap4 (primary PCR) and Akt-3sp2/Akt-3ap5 (nested PCR) as primers. The resulting 1200 bp PCR fragment was then cloned in the TA-cloning vector pCR2.1 (original TA cloning kit, Invitrogen BV, Leek, The Netherlands) and the inserts of several clones were completely sequenced. One clone containing an insert with the confirmed sequence (hAkt-3/pCR2.1) was used for subsequent subcloning to the mammalian expression vector pCDNA-3 (Invitrogen), yielding construct hAkt-3/pCDNA-3. In order to make a construct coding for a COOH-terminal tagged Akt-3 protein, a fragment of 553 bp was amplified from plasmid Akt-3/pCDNA-3 using an antisense primer incorporating a *Xho*I restriction site and the sequence coding for a hemagglutinin (HA) tag (YPYDVPDYA) after amino acid 479 of the Akt-3 sequence. This fragment was recloned into plasmid hAkt-3/pCDNA-3 using *Bst*EII and *Xho*I restriction sites yielding construct HA-hAkt-3/pCDNA-3.

Constructs and mutants for *E. coli* expression of Akt-3.

5 In order to express the human Akt-3 protein in *E. coli*, the complete Akt-3 coding sequence was amplified from plasmid hAkt-3/pCR2.1 using primers introducing a *EcoRI* restriction site and a *XhoI* restriction site at the 5' and 3' ends, respectively. This PCR fragment was
10 cloned as a *EcoRI/XhoI* fragment in vector pGEX-4T-3 (Amersham Pharmacia Biotech, Uppsala, Sweden) yielding construct hAKT-3(WT)/pGEX-4T-3, and the sequence of the insert was confirmed by sequence analysis.

15 Mutants of this construct were made using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The T305D mutant (construct hAKT-3(T305D)/pGEX-4T-3) was created by mutating ACA at
20 position 923-925 to GAC, resulting in a Thr³⁰⁵ to Asp mutation in the resulting protein. The S472D mutant (construct hAKT-3(S472D)/pGEX-4T-3) was created by changing TC at position 1404-1405 to GA using PCR with a long antisense primer incorporating the change,
25 resulting in a Ser⁴⁷² to Asp mutation in the resulting protein. A double mutant was also constructed by site-directed mutagenesis on hAKT-3(S472D)/pGEX-4T-3 and contained both these mutations (construct hAKT-3(T305D/S472D)/pGEX-4T-3). The inserts of all
30 resulting constructs were confirmed by complete sequence analysis. The fusion proteins resulting from expression of these constructs in *E. coli* contain a GST moiety coupled to the NH₂-terminus of the human Akt-3 sequence.

Expression in Cos-7 cells

Akt-3 was transiently expressed in Cos-7 by calcium phosphate transfection of the cells with the construct HA-hAkt-3/pcDNA-3. The cells were stimulated with 10 ng/ml IGF-1 for 30 minutes, lysed and Akt-3 immunoprecipitated with mAb 12CA5. Akt-3 activity was assessed as described below.

Expression and assay of wild-type and mutant Akt-3 in *E. coli*.

The expression constructs were transformed into *E. coli* strain BL21 DE3 and GST-fusion proteins of wild-type and mutated Akt-3 were purified on glutathione sepharose according to the manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). The protein eluted from the beads was stored in 50% glycerol at -20°C. Akt activity was assessed by incubating 0.8 µg of the purified enzyme for 30 minutes at room temperature (unless otherwise indicated) in a buffer containing 10 mM HEPES, 10mM MgCl₂, 1 mM DTT, 0.1 mg/ml histone H2B at pH 7.0, in a total volume of 25µl and containing 10 µCi [γ-³²P]-ATP (6000 Ci/mmol). Initial experiments indicated that the reaction was linear with time for at least 45 minutes. The reaction was stopped by the addition of 25 µl sample buffer for SDS-PAGE. The results were quantified on a phosphorimager following SDS-PAGE on a 15% (w/v) acrylamide gel.

Chromosomal mapping studies

Chromosomal mapping studies were carried out by SeeDNA

Biotech Inc, Toronto, Canada using fluorescent *in situ* hybridisation (FISH) analysis essentially as described (Heng et al., 1992; Heng & Tsui, 1993). Briefly, human lymphocytes were cultured at 37°C for 68-72 h before treatment with 0.18 mg/ml 5-bromo-2'-deoxyuridine (BrdU) to synchronize the cell cycle in the cell population. The synchronized cells were washed and recultured at 37°C for 6 h. Cells were harvested and slides were prepared using standard procedures including hypotonic treatment, fixation and air-drying. A cDNA probe for Akt-3 (1.44 kb *Eco*RI fragment of clone hAkt-3/pcDNA-3) was biotinylated and used for FISH detection. Slides were baked at 55°C for 1 h, treated with RNase and denatured in 70% (v/v) formamide in 2x NaCl/Cit (0.3 M NaCl, 0.03 M disodium citrate, pH 7.0) for 2 min at 70°C followed by dehydration in ethanol. Probes were denatured prior to loading on the denatured chromosomal slides. After overnight hybridisation, slides were washed and FISH signals and the 4',6-diamidino-2-phenylindole banding pattern were recorded separately on photographic film, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposition of FISH signals with 4',6-diamidino-2-phenylindole banded chromosomes (Heng & Tsui, 1993).

Northern blot analysis.

Northern blots containing 2 µg of poly(A)-rich RNA derived from different human tissues (Clontech Laboratories, Palo Alto, CA, USA) were hybridised according to the manufacturer's instructions with a α-³²P-dCTP random-priming labelled (HighPrime kit, Boehringer Mannheim) 454 bp *Not*I-*Xba*I Akt-3 fragment (nucleotides 1404 to 1857) corresponding to part of

the 3' untranslated sequence.

Reverse transcription (RT)-PCR analysis

Oligonucleotide primers were designed for the specific
5 PCR amplification of a fragment from Akt-3. These
primers were Akt-3sp2 = 5'-GGG AGT CAT CAT GAG CGA TGT
TAC C-3' (sense primer) and Akt-3ap1 = 5'- GGG TTG TAG
AGG CAT CCA TCT CTT CC -3' (antisense primer),
yielding a 425 bp product. PCR amplifications for
10 human glyceraldehyde-3-phosphate dehydrogenase (G3PDH)
were performed on the same cDNA samples as positive
controls using G3PDH primers 5'-TGA AGG TCG GAG TCA
ACG GAT TTG GT-3' (sense primer) and 5'-CAT GTG GGC CAT
GAG GTC CAC CAC-3' (antisense primer), yielding a 1000
15 bp fragment. These primers were used for PCR
amplifications on Multiple Tissue cDNA panels
(Clontech Laboratories) and on cDNA prepared from
tumor cell lines. For the preparation of tumor cell
cDNA, cells were homogenised and total RNA prepared
20 using the RNeasy Mini kit (Qiagen GmbH, Hilden,
Germany) according to manufacturer's instructions. 1
 μ g of total RNA was reverse transcribed using
oligo(dT)₁₅ as a primer and 50 U of Expand™ Reverse
Transcriptase (Boehringer Mannheim, Mannheim, Germany)
25 according to the manufacturer's instructions. PCR
reactions with Akt-3-specific or G3PDH-specific
primers were then performed on 1 μ l of cDNA. Images of
the ethidium bromide stained gels were obtained using
the Eagle Eye II Video system (Stratagene, La Jolla,
30 CA, USA) and PCR bands analysed using the EagleSight
software.

RESULTS

Molecular cloning of human Akt-3.

5 Similarity searching of the LifeSeqTM and EMBL
databases using the rat Akt-3 sequence as a query
sequence yielded several human EST sequences which
encoded part of the human homologue of rat Akt-3.
Using the DNA sequence information in the databases,
10 we were able in subsequent 3' RACE experiments to
deduce the complete cDNA sequence for the human Akt-3
(Figure 1). The obtained cDNA sequence encoded a
protein of 479 amino acid residues with a calculated
molecular mass of 55770 Da. The first 451 amino acids
15 of the human Akt-3 protein contain only two
differences to the corresponding rat sequence (Konishi
1995) - Asp (rat) to Gly (human) at position 10 and
Pro (rat) to Ala (human) at position 396 and encode a
pleckstrin homology domain, a kinase domain and a
20 COOH-terminal 'tail'. The predicted Akt-3 (Figure 2)
protein shows significant similarity with Akt-1 (Jones
et al, 1991; 83.6% identity; 87.8% similarity) and
with Akt-2 (Cheng 1992; 78% identity; 84.3%
similarity). The COOH-terminal 'tail' has been observed
25 in both human and rat Akt-1 and Akt-2 proteins, but it
is apparently truncated in the only other reported
Akt-3 sequence (rat Akt-3, Konishi 1995; accession
number D49836). 3' RACE experiments performed on human
cDNAs derived from different tissues did not yield
30 evidence for the existence of a shorter form of Akt-3
that would be analogous to the rat Akt-3 (data not
shown). The tail in human Akt-3 comprises 28 amino
acid residues (YDEDGMDCMDNERRPHFPQFSYSASGRE) that
replace 3 amino acid residues in the rat sequence
35 (CPL). The tail in human Akt-3 contains a serine

residue at position 472 (shown in bold) that corresponds to Ser⁴⁷³ in Akt-1 or Ser⁴⁷⁴ in Akt-2. Phosphorylation of Ser⁴⁷³ and Ser⁴⁷⁴ has previously been implicated in the activation of Akt-1 and Akt-2 ,
5 respectively (Alessi 1996; Meier 1997). Thr³⁰⁸ (in the kinase domain) has also been implicated in the activation of Akt-1 and this residue is also conserved in human Akt-3 (Thr³⁰⁵).

10 **Characterisation of Akt-3 activity.**

To characterise the enzymatic activity of Akt-3, we expressed and purified the recombinant enzyme as a GST fusion protein. Analysis of the purified product by
15 SDS-PAGE indicated the protein was apparently > 90% pure. The purified enzyme was able to phosphorylate histone H2B (figure 3), and no phosphorylation was observed using recombinant GST alone. Previously, the enzymatic activity of Akt-1 has been shown to be
20 increased by phosphorylation of Thr³⁰⁸ and Ser⁴⁷³, and mutation of both these residues to Asp (to mimic phosphorylation) synergistically activates Akt-1 (Alessi et al., 1996). To investigate whether Akt-3 is similarly regulated, GST-fusion proteins in which
25 either Thr³⁰⁵ or Ser⁴⁷² (corresponding to Thr³⁰⁸ and Ser⁴⁷³ in Akt-1) or both Thr³⁰⁵ and Ser⁴⁷² had been mutated to Asp were expressed and assayed in comparison to the wild-type enzyme. Mutation of Thr³⁰⁵ to Asp ("T305D") resulted in a 2.0-fold increase in the initial rate of
30 phosphorylation of histone H2B, whereas mutation of Ser⁴⁷² to Asp("S472D") increased the initial rate only 1.4 fold (Figure 3A). When both Thr³⁰⁵ and Ser⁴⁷² ("T305D,S472D") were mutated to Asp, a 3.2-fold increase in the initial phosphorylation rate was
35 observed.

To confirm that extracellular stimuli can activate Akt-3 in mammalian cells, Cos-7 cells were transfected with a cDNA encoding Akt-3 fused to a HA tag. Akt-3 activity in HA immunoprecipitates was increased 1.5 and 1.9 fold (n=2) following stimulation with IGF-1 (10 ng/ml) (Fig 3B).

To characterise human Akt-3 further, we investigated the ability of a range of Ser/Thr kinase inhibitors to inhibit Akt-3. These included Go 6976, GF-109203X (both protein kinase C (PKC) inhibitors); H-85, H-88, H-89 and KT5720 (protein kinase A (PKA) inhibitors), KN-62 (Ca²⁺/Calmodulin dependent kinase inhibitor) and PD 98059 (MEK inhibitor). when tested at a concentration of 10 μ M these compounds had no significant effect on the activity of the T305D,S472D variant Akt-3. However, the broad spectrum kinase inhibitor staurosporine (IC₅₀= 2.0 \pm 0.3 μ M) and the PKC inhibitor Ro 31-8220 (IC₅₀=3.2 \pm 1.0 μ M) inhibited the T305D,S472D variant of Akt-3 (Figure 4).

Chromosomal localisation of Akt-3.

The complete coding sequence of Akt-3 was used as a probe for FISH analysis. Under the conditions used, the hybridisation efficiency was approximately 75% for this probe (among 100 checked mitotic figures, 75 of them showed signals on one pair of the chromosomes). Since the DAPI-banding was used to identify the specific chromosome, the assignment between the signal from the probe and the long arm of chromosome 1 was obtained. The detailed position was further determined in the diagram based upon the summary from 10 photographs (Figure 5A). There was no additional locus picked by FISH detection under the conditions used,

therefore, we conclude that Akt-3 is located at human chromosome 1, region q43-q44. An example of the mapping results is presented in Figure 5 B.

5 **Tissue distribution of Akt-3 mRNA.**

Northern blot analysis was performed on mRNA derived from different human tissues. Akt-3 mRNA was detected as two transcripts of approximately 4.5 kb and 7.5 kb, showing similar patterns of expression (Fig. 6A). Akt-3 mRNA was expressed in a range of tissues, most prominently in brain. Similarly, rat Akt-3 was detected as multiple transcripts most highly expressed in brain (Konishi et al., 1995). The weakest expression of Akt-3 was observed in two insulin-responsive tissues, skeletal muscle and liver. Akt-3 was also expressed in a number of cancer cell lines including SW480 colorectal adenocarcinoma, A549 lung carcinoma and G361 Melanoma (data not shown).

20 To confirm the Northern blot analysis, PCR reactions were performed with Akt-3 specific and G3PDH-specific (internal control) primers on cDNAs derived from different human tissues (Fig. 6B). The Akt-3 message was present in every tissue tested, since a specific 425 bp fragment was amplified in every cDNA after 30 cycles of PCR. Akt-3 mRNA expression was high in placenta, ovary and spleen. Moderate expression was seen in brain, heart, kidney, colon, prostate, small intestine and testis. Lowest expression was in liver, lung, pancreas, skeletal muscle, peripheral blood leukocytes and thymus. In tumor cell lines (Figure 6C), Akt-3 mRNA expression was relatively high in HT-1080 bone fibrosarcoma cells, in SaOS-2 osteosarcoma and in JURKAT T-cell leukemia cells (Akt-3 band

detectable after 30 cycles of PCR). Caco-2 colorectal adenocarcinoma, T84 colorectal carcinoma, MCF-7 breast adenocarcinoma and SK-N-MC neuroblastoma cells show Akt-3 mRNA expression after 35 cycles of PCR. In T-47D breast ductal gland carcinoma and HepG2 hepatoblastoma, expression of Akt-3 mRNA is very low or absent (no signal detectable after 35 cycles of PCR).

Akt-1 and Akt-2 have been identified in several species. Human (Jones et al., 1991; Coffey et al 1991), mouse (Bellacosa et al., 1993) and bovine (Coffey & Woodgett, 1991) Akt-1 clones have been reported, whereas human (Cheng et al., 1992) mouse (Altomare et al., 1995) and rat (Konishi et al., 1994) clones of Akt-2 have been identified. However, Akt-3 has only been previously identified in rat (Konishi et al, 1995). We have identified the human isoform of Akt-3. Although human Akt-3 shows considerable similarity to human Akt-1 and Akt-2, the discovery of human Akt-3 is particularly significant because the cDNA sequence encodes a COOH-terminal 'tail' which includes a phosphorylation site implicated in the activation of Akt-1 and Akt-2 (Alessi et al., 1996; Meier et al., 1997). This tail is absent from the predicted rat amino acid sequence. Human Akt-3 appears to be activated by phosphorylation in a similar fashion as Akt-1 and Akt-2. However, its expression profile suggests that the principal function of this enzyme is not in regulating responses to insulin.

The sequence we have identified represents the human homologue of Akt-3. This assignment is based on the >99% identity between the rat and human Akt-3 protein sequences. With the exception of the COOH-terminal

tail seen in human Akt-3, there are only 2 amino acid differences (Gly¹⁰ and Ala³⁹⁶ in human Akt-3) between the rat and human Akt-3 proteins. Alignment of all the previously described Akt sequences demonstrates that Gly¹⁰ and Ala³⁹⁶ in the human protein correspond to Gly and Ala residues respectively in the Akt-1 and Akt-2 sequences identified from other species. Further evidence that we have identified the Akt-3 isoform comes from the presence of isotype-specific sequences represented by human Akt-3 residues 47-49 (LPY), 118-122 (NCSPT) and 139-141 (HHK). For each isotype, these sequences are conserved between species, but differ between the isotypes.

The human Akt-3 cDNA sequence was predicted to encode a NH₂-terminal pleckstrin homology (PH) domain (Musacchio et al., 1993) and a COOH-terminal kinase domain. A striking difference between the human and rat Akt-3 protein sequence (Konishi, 1995) is the presence of a COOH-terminal 'tail' comprising 74 residues after the kinase domain. The last 28 amino acid residues in human Akt-3 are absent from the rat Akt-3 sequence. We were unable to identify human cDNA sequences that encoded a similar truncation, despite conducting RACE experiments using cDNA from several different human tissues. The region in human Akt-3 that is absent from rat Akt-3 encompasses a stretch of 10 residues (residues 467-476 in human Akt-3) which are identical to the corresponding region of human Akt-1 and Akt-2. This suggests that the tail observed in human Akt-3 is authentic. The significance of the difference observed in the rat Akt-3 tail region remains to be investigated. However, the human Akt-3 COOH-terminal sequence includes Ser⁴⁷², which corresponds to Ser⁴⁷³ in Akt-1. Phosphorylation of

Ser⁴⁷³ has been shown to lead to a 5-fold increase in the activity of Akt-1, whereas a 20-25 fold increase of Akt-1 activity is observed if both Ser⁴⁷³ and Thr³⁰⁸ are phosphorylated (Alessi et al., 1996). Thus, our
5 observation that Ser⁴⁷² is present in human Akt-3 is significant, because it suggests that human Akt-3 is potentially regulated in a manner similar to Akt-1 and Akt-2. Whether rat Akt-3 is regulated in a different fashion remains to be resolved.

10

The kinase and PH domains in Akt-3 show homology to the consensus PH and kinase domain sequences (Musacchio et al, 1993; Hanks & Hunter 1995). The PH domain of human Akt-3 is 77% and 86% identical to the
15 PH domains in Akt-1 and Akt-2, respectively, while the kinase domain of Akt-3 is 88% and 87% identical to the kinase domain of Akt-1 and Akt-2, respectively. The high conservation of the PH domain may indicate an Akt-specific function, because PH domains are often
20 highly divergent (Musacchio et al, 1993). Apart from binding phosphoinositides, the PH domain of Akt has been shown to mediate interactions between Akt and PKC (Konishi, 1995) as well as directing the formation of multimeric Akt complexes (Datta et al, 1995). In
25 contrast, the region between the PH domain and the kinase domain is poorly conserved between the human Akt-1, Akt-2 and Akt-3 sequences, and this region is also important for mediating the formation of multimeric Akt complexes (Datta et al, 1995). This
30 raises an interesting issue - whether the sequence NH₂-terminal to the kinase domain of Akt-3 mediates the interaction with binding partners that are unique to Akt-3 or that bind to multiple Akt isoforms.

35 To verify that the predicted kinase domain was

catalytically active, we expressed Akt-3 as a GST fusion protein in *E. Coli*. The purified protein was able to phosphorylate an exogenous substrate, whereas no catalytic activity was observed using GST in place of GST-Akt-3. To confirm that Akt-3 is indeed regulated in a manner akin to Akt-1 and Akt-2, we mutated Thr³⁰⁵ and Ser⁴⁷³, either separately or jointly, to Asp. This strategy has previously been shown to faithfully mimic the effect of phosphorylation of these residues in Akt-1 (Alessi et al., 1996). Mutation of either of these residues resulted in increased activity, although the increase was less than that observed with Akt-1 (Alessi et al., 1996). Additionally, we did not observe a synergistic activation of Akt-3 by mutation of both Thr³⁰⁵ and Ser⁴⁷³. In contrast, when both the corresponding residues were simultaneously mutated to Asp in Akt-1, synergistic activation was observed (Alessi et al., 1996). The apparent quantitative differences between Akt-1 and Akt-3 may reflect true differences in the regulation of these two isoforms, or it may be due to other factors such as the different expression system used. In the present study Akt-3 was expressed as a GST fusion protein in *E. Coli*, whereas Akt-1 activity was studied using an HA-tagged protein expressed in COS cells. Nevertheless, our results demonstrate that Akt-3 is qualitatively regulated in a fashion similar to Akt-1. Previous work has also shown that activation of Akt is dependent upon PI 3-kinase to generate 3-phosphoinositides that bind the PH domain of Akt, promote translocation of Akt to the plasma membrane and facilitate the phosphorylation of Akt by upstream kinases (reviewed in Alessi & Cohen, 1998; Coffey et al., 1998). Our observation that the T305D/S472D mutant of Akt-3 is more active than the wild type

enzyme (Figure 3), when measured in the absence of 3-phosphoinositides, suggests that after phosphorylation Akt-3 becomes (at least partially) independent of phosphoinositide binding.

5

The structure of the catalytic domain of Akt is closely related to Protein kinase A and Protein kinase C. Indeed, a BLAST search of the SwissProt data base revealed that the most closely related kinases (other
10 than the different Akt isoforms) include several protein kinase C isozymes. This prompted us to investigate whether existing inhibitors of PKA or PKC, as well as other serine/threonine kinase inhibitors, could be used as inhibitors of Akt-3. Of the compounds
15 tested, only staurosporine and the structurally related compound Ro 31-8220 both potently inhibited Akt-3. Staurosporine is a non-selective kinase inhibitor, whereas Ro 31-8220 is a more selective PKC inhibitor (Davis, 1992). Although Ro 31-8220 is an
20 approximately 100-fold more potent ($IC_{50} \approx 10$ nM; Davis, 1992) inhibitor of PKC than of Akt-3, this observation cautions that experiments using high concentrations of Ro 31-8820 may affect Akt-3. In contrast to staurosporine and Ro 31-8220, two other PKC inhibitors
25 and three other PKA inhibitors did not inhibit Akt-3. This suggests that although Akt-3 is closely related in sequence to PKC, it may be possible to find selective inhibitors of Akt.

30 The observation that Akt-3 is activated by IGF-1 suggests that Akt-3 may play a role in regulating cell survival. Akt-3 potentially may suppress apoptosis in tumor cells. One concern in using Akt as a target for drug development in cancer is that Akt plays a role in
35 insulin signalling (reviewed in Sheperd et al, 1998).

Thus, inhibitors of Akt may induce symptoms observed in patients with diabetes. One solution that has been proposed is to develop selective inhibitors of Akt-2 (Walker et al, 1998). This is based in part on the
5 observation that Akt-1 is strongly activated by insulin in rat hepatocytes and skeletal muscle, whereas Akt-2 is only weakly activated by insulin in these tissues. However, rat Akt-3 appears to be even more weakly activated by insulin in these tissues
10 (Walker et al, 1998), and in this study we have shown that Akt-3 mRNA is expressed only at low levels in human liver and skeletal muscle, which are insulin responsive tissues. This suggests that selective inhibitors of Akt-3 could have even less potential to
15 cause symptoms similar to those seen in patients with diabetes than do inhibitors of Akt-2. The localisation of human Akt-3 to human chromosome 1q43-44 is also interesting, as patients with haematological cancers have been reported with chromosomal abnormalities in
20 this region (Mitelman et al, 1997). Although the significance of the latter observation is debatable, as chromosomal abnormalities at numerous loci have been observed in patients with haematological cancers, the results presented here indicate that Akt-3 may
25 prove to be an important target for the development of novel therapeutics for the treatment of cancer.

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CLAIMS

1. A nucleic acid molecule encoding human Akt-3 or a functional equivalent or bioprecursor thereof,
5 comprising the amino acid sequence illustrated in Figure 2.
2. A nucleic acid molecule according to claim 1 which is a DNA molecule, and preferably cDNA.
- 10 3. An nucleic acid molecule according to claim 1 or 2 comprising the nucleotide sequence illustrated in Figure 1.
- 15 4. An antisense molecule capable of hybridising to the molecule according to any of claims 1 to 3 under high stringency conditions.
- 20 5. Human Akt-3 or a functional equivalent, derivative or bioprecursor thereof, comprising an amino acid sequence as illustrated in Figure 2.
- 25 6. Human Akt-3 or a functional equivalent, derivative or bioprecursor thereof encoded by a nucleic acid molecule according to any of claims 1 to 3.
- 30 7. Human Akt-3 according to claim 6 comprising the amino acid sequence as illustrated in Figure 2.
8. An expression vector comprising a nucleic acid molecule according to claim 2 or 3.
- 35 9. An expression vector according to claim 8 comprising an inducible promoter.

10. An expression vector according to claim 8 or 9 comprising a sequence encoding a reporter molecule.

5 11. A nucleic acid molecule according to any of claims 1 to 4 for use a medicament.

12. A nucleic acid molecule according to any of claims 1 to 4 in the preparation of a medicament for treating cancer.

10 13. Human Akt-3 according to any of claims 5 to 7 for use as a medicament.

15 14. Use of human Akt-3 according to any of claims 5 to 7 in the preparation of a medicament for treating cancer.

20 15. A pharmaceutical composition comprising a nucleic acid molecule according to any of claims 1 to 4 or a human Akt-3 according to any of claims 5 to 7 together with a pharmaceutically acceptable carrier diluent or excipient therefor.

25 16. A host cell or organism, transformed or transfected with an expression vector according to any of claims 8 to 10.

30 17. A transgenic cell, tissue or organism comprising a transgene capable of expressing human Akt-3 according to any of claims 5 to 7.

18. A human Akt-3 protein expressed from the cell or organism according to claim 16 or 17.

35 19. An antibody capable of binding to human Akt-3 or

an epitope thereof according to any of claims 5 to 7.

20. An antibody according to claim 19 which is a monoclonal antibody.

5

21. An antibody according to claim 19 or 20 for use as a medicament.

10 22. Use of an antibody according to claim 19 or 20 in the preparation of a medicament for treating cancer, or other diseases or conditions associated with human Akt-3 expression.

15 23. A kit for detecting human Akt-3 in a sample having a sequence according to any of claims 5 to 7, which kit comprises an antibody according to claim 19 or 20 and means for contacting said antibody with said sample.

20 24. A method of identifying compounds which selectively inhibit human Akt-3 mediated promotion of cell survival upon withdrawal of survival factors, said method comprising:

- 25 i) providing on the one hand a cell that survives in the presence of a survival factor but not in the absence of said survival factor unless said cell is transformed with an expression vector according to any of claims 8 to 10, in
30 addition to providing a cell which in the presence of said survival factor has not been transformed with said vector,
ii) contacting each of said cells with a test
35 compound to ensure said compound is not toxic to said cells, iii) subsequently

5 contacting each of said cells with the test
 compound following the removal of said cells
 from said survival factors, wherein death of
 said cell when transformed with said
 expression vector is indicative of selective
 inhibition of said compound on the survival
 promoting Akt-3 pathway or a parallel
 pathway.

10 25. A compound identifiable according to the method
 of claim 24.

 26. A compound according to claim 25 for use as a
 medicament.

15 27. Use of a compound according to claim 25 in the
 manufacture of a medicament for treating diseases
 associated with human Akt-3 expression.

20 28. A method of identifying agents which influence
 the activity of a human Akt-3 protein according to any
 of claims 5 to 7, said method comprising contacting
 said human Akt-3 with a substrate therefor in the
 presence of a test compound and a phosphate source,
25 and monitoring for any phosphorylation of said
 substrate.

 29. A method according to claim 28 wherein said Akt-3
 is provided as a fusion or epitope tagged protein
30 having a domain capable of phosphorylating a known
 substrate.

 30. A method according to claim 26 wherein said Akt-3
 is provided as a fusion molecule of GST and human Akt-
35 3 according to the invention.

31. A method of identifying agents which influence the activity of human Akt-3 protein according to any of claims 5 to 7, said method comprising contacting a phospholipid or a surrogate or functional equivalent thereof, with a PH domain of a human Akt-3 protein according to any of claims 5 to 7 in the presence of an agent to be tested and monitoring for any binding of said phospholipid, surrogate or functional equivalent thereof with said PH domain of said Akt-3 protein.

32. A method according to claim 31 wherein said phospholipid comprises phosphatidylinositol 3, 4, 5 - triphosphate.

33. An agent identifiable according to the method of claim 31 or 32.

34. An agent according to claim 33 for use as a medicament.

35. Use of an agent according to claim 33 in the preparation of a medicament for treating diseases associated with human Akt-3 expression.

36. A method of treating diseases associated with human Akt-3 expression said method comprising administering to an individual suffering from said disease a compound that inhibits the function or expression of human Akt-3 according to any of claims 5 to 7, in a sufficient concentration to reduce the symptoms of said disease.

37. A method according to claim 36 wherein said compound is any of an antisense molecule according to

claim 4, an antibody according to claim 20 or 21, a compound according to claim 23 or an agent according to claim 33.

- 5 38. A method for making a pharmaceutical formulation for the treatment of diseases associated with human Akt-3 expression, said method comprising:
- 10 a) contacting candidate compounds with a host cell that expresses human Akt-3 protein,
- b) selecting a compound identified in step a) which binds to human Akt-3 protein,
- c) manufacturing bulk quantities of the compound selected in step b), and
- 15 d) formulating the compound manufactured in step c) in a pharmaceutically acceptable carrier.

M S D V T I V K E G W V O K R G E 17
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 Y I K N W R P R Y F L L K T D G S F I G 37
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 Y K E K P Q D V D L P Y P L N N F S V A 57
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 R E 479
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 Akt-2 : MNEVSIVKEGMLHKRGEXIKIMRERYFLKSDGSEFIGYKEKPEAPDCTIPEDNNFSVACCOLMKTERPRPENTFTIRCLQMTTIVERTFPHV : 90
 Akt-3 : MEDVTIVKEGMLVKRGEXIKIMRERYFLKTDGSEFIGYKEKPEQDVCLP-YELNNFSVACCOLMKTERPRPENTFTIRCLQMTTIVERTFPHV : 89

Akt-1 : EUPERREBMTTAICTVAJGLIKQE--EENDFRSGSPEDNBSGAEMEVSIAKPMHRVUTMNEFEPYLLKLLGKGTGFKVILVKEKALGKSNYYAM : 178
 Akt-2 : DBEDREEMMRALQWANSUKORAPGEDPMDYKCESPEDSSTTEEMEVAVSARAKUTMNDPFPYLLKLLGKGTGFKVILVKEKALGKSNYYAM : 180
 Akt-3 : LAFERREBMTTAICAVAJPLQOE--ERMNCSPTSOIDLIGEEMDAETTHG-PKTMNDPFPYLLKLLGKGTGFKVILVKEKALGKSNYYAM : 176

Akt-1 : KILLKREVTIAKDEVAHTTTEMRVTQNSRHFFLTALKYSEFOTDRLCFVMEYANGGELIFFHLSRERVFEEDRARFYGAIEIVSALTYLHSEK : 268
 Akt-2 : KILLKREVTIAKDEVAHTVTEMRVTQNSRHFFLTALKYAFOTDRLCFVMEYANGGELIFFHLSRERVFEEDRARFYGAIEIVSALTYLHS-R : 269
 Akt-3 : KILLKREVTIAKDEVAHTTTEMRVTQNSRHFFLTSLKYSEFOTDRLCFVMEYANGGELIFFHLSRERVFEEDRARFYGAIEIVSALTYLHS-G : 265

Akt-1 : NUVYRDJIKLENMLDKDGHIKITDDEGICKEGIKDQATMKTFCCGTPEYLAPEVLEDNDYGRAVDMMGLGVVMYEMMCGRLPFYMQDHEKLEF : 358
 Akt-2 : DUVYRDJIKLENMLDKDGHIKITDDEGICKEGISDQATMKTFCCGTPEYLAPEVLEDNDYGRAVDMMGLGVVMYEMMCGRLPFYMQDHEKLEF : 359
 Akt-3 : KIVYRDJIKLENMLDKDGHIKITDDEGICKEGITDAATMKTFCCGTPEYLAPEVLEDNDYGRAVDMMGLGVVMYEMMCGRLPFYMQDHEKLEF : 355

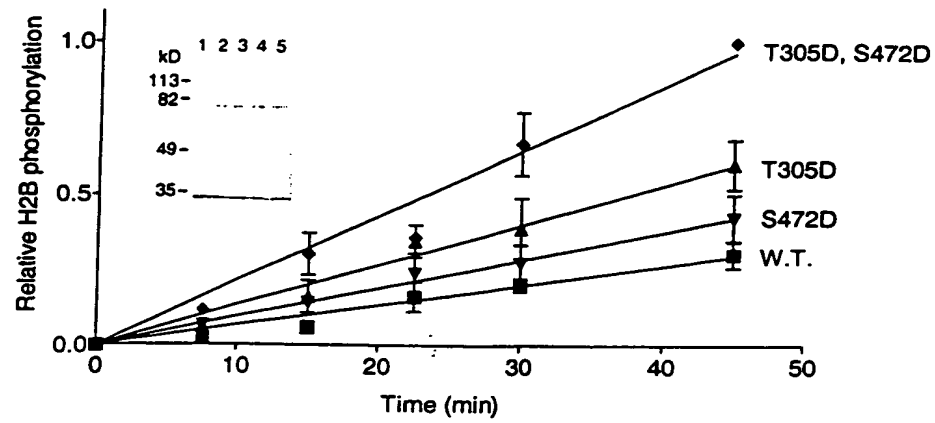
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 Akt-2 : ELLIMEDIRPPTLSPEAKSLLSGLLKKDKQKQRLGGSESDAKEMCHRRFPFLSINMQDVCKKLLPPFPKQVTSNDTRYFDEEPTACMLT : 449
 Akt-3 : ELLIMEDIRPPTLSPEAKSLLSGLLKKDKQKQRLGGSESDAKEMCHRRFPFLSINMQDVCKKLLPPFPKQVTSNDTRYFDEEPTACMLT : 445

Akt-1 : ITPPDQDSS--TEGVDSERPPEHFPQFSYSASGTA : 480
 Akt-2 : ITPPDQDSS--IGLLELDQTHFPQFSYSASGTA : 481
 Akt-3 : ITPPEKTEDECDQDQENERPPEHFPQFSYSASGTA : 479

Fig 2

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A



B

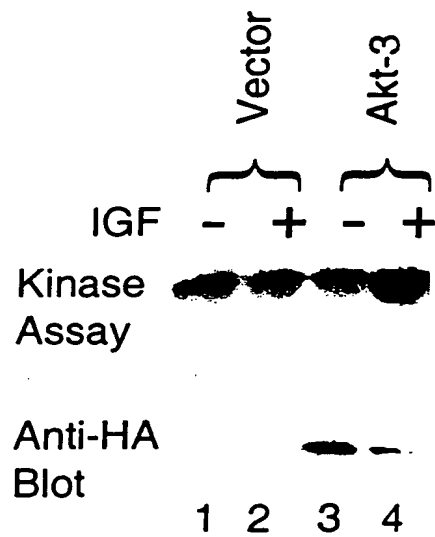


Fig 3

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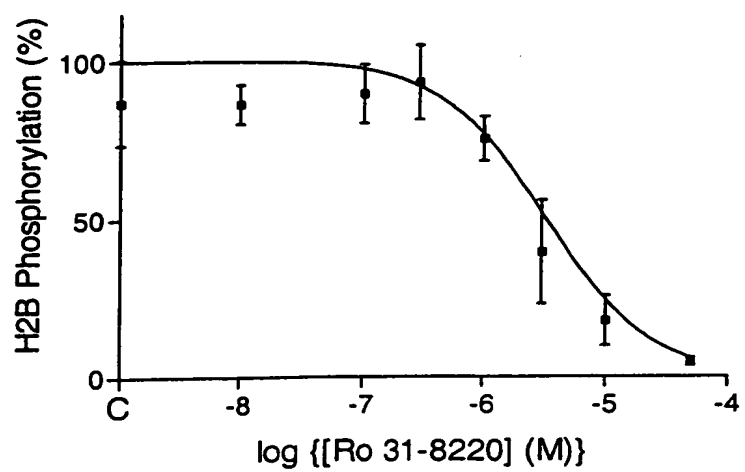
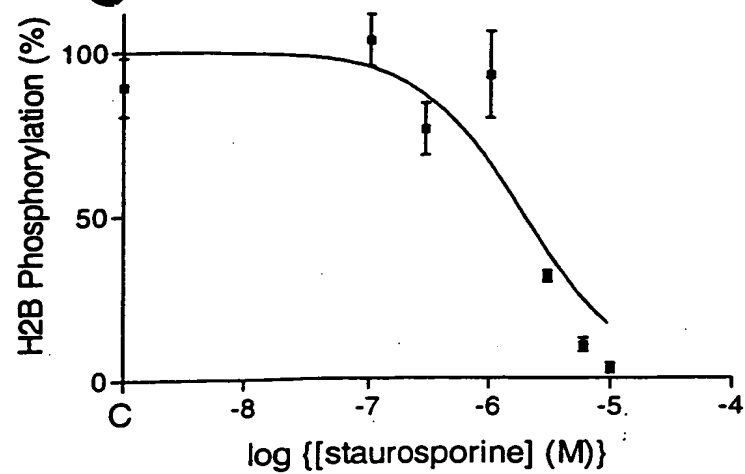
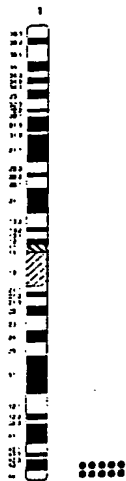


Figure 4.

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A



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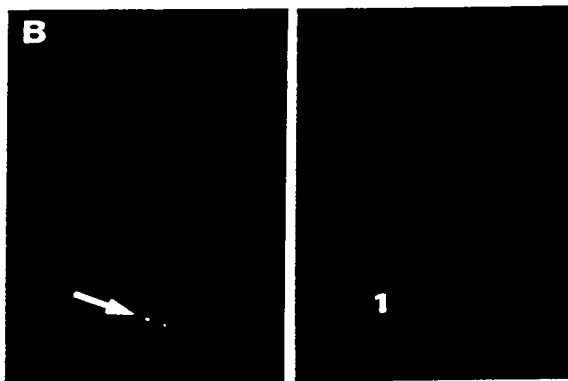


Fig 5

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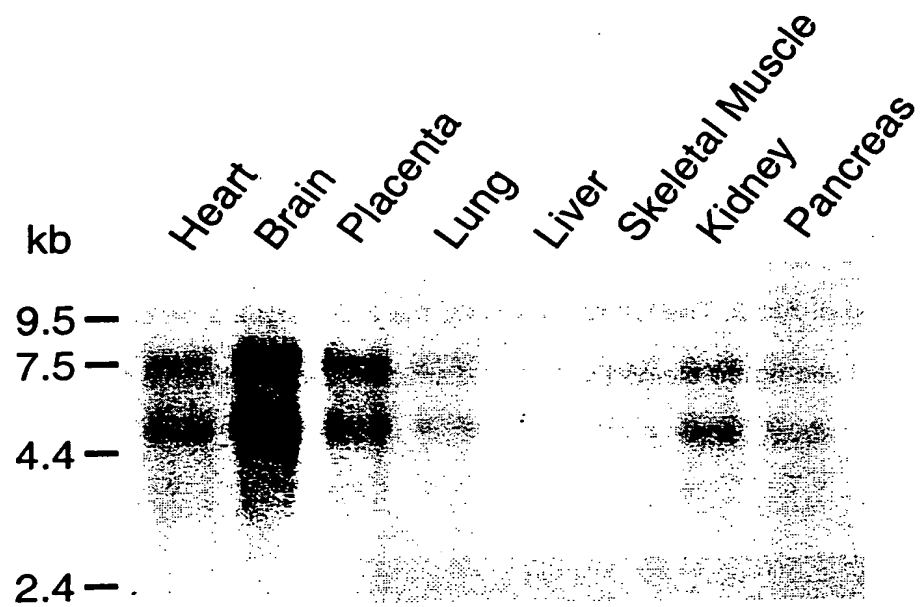


Figure 6A

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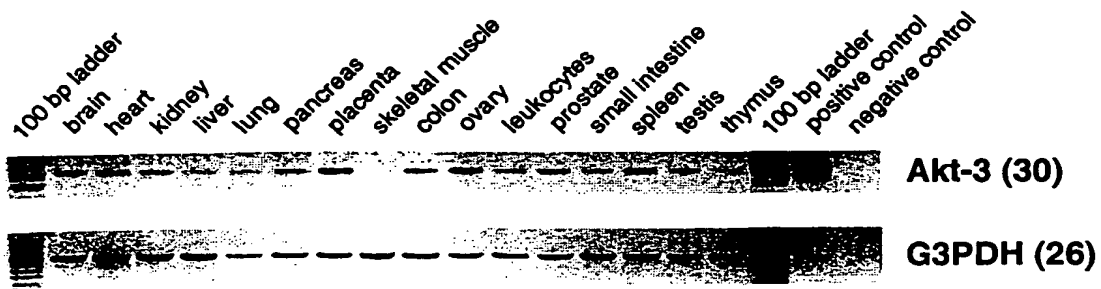


Figure 6B

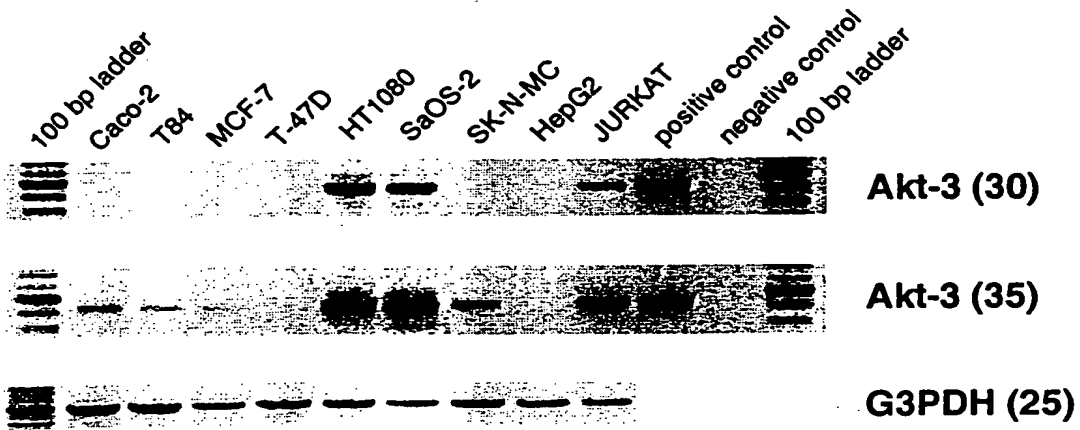


Figure 6C

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